

# ATPase activity of the sulfonylurea receptor: a catalytic function for the $K_{ATP}$ channel complex

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**ABSTRACT** ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels are unique metabolic sensors formed by association of Kir6.2, an inwardly rectifying  $K^+$  channel, and the sulfonylurea receptor SUR, an ATP binding cassette protein. We identified an ATPase activity in immunoprecipitates of cardiac  $K_{ATP}$  channels and in purified fusion proteins containing nucleotide binding domains NBD1 and NBD2 of the cardiac SUR2A isoform. NBD2 hydrolyzed ATP with a twofold higher rate compared to NBD1. The ATPase required  $Mg^{2+}$  and was insensitive to ouabain, oligomycin, thapsigargin, or levamisole. K1348A and D1469N mutations in NBD2 reduced ATPase activity and produced channels with increased sensitivity to ATP.  $K_{ATP}$  channel openers, which bind to SUR, promoted ATPase activity in purified sarcolemma. At higher concentrations, openers reduced ATPase activity, possibly through stabilization of MgADP at the channel site. K1348A and D1469N mutations attenuated the effect of openers on  $K_{ATP}$  channel activity. Opener-induced channel activation was also inhibited by the creatine kinase/creatine phosphate system that removes ADP from the channel complex. Thus, the  $K_{ATP}$  channel complex functions not only as a  $K^+$  conductance, but also as an enzyme regulating nucleotide-dependent channel gating through an intrinsic ATPase activity of the SUR subunit. Modulation of the channel ATPase activity and/or scavenging the product of the ATPase reaction provide novel means to regulate cellular functions associated with  $K_{ATP}$  channel opening.—Bienengraeber, M., Alekseev, A. E., Abraham, M. R., Carrasco, A. J., Moreau, C., Vivaudou, M., Dzeja, P. P., Terzic, A. ATPase activity of the sulfonylurea receptor: a catalytic function for the  $K_{ATP}$  channel complex. *FASEB J.* 14, 1943–1952 (2000)

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subunits combine to form functional channels (1–4). In this way, ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels are formed by association of an inwardly rectifying  $K^+$  channel, Kir6.2, with the ATP binding cassette (ABC) protein, the sulfonylurea receptor SUR (5, 6). Originally discovered in the heart (7),  $K_{ATP}$  channels transduce cellular metabolic signals into membrane potential changes and regulate critical cellular functions, including cytoprotection (8–11). Although SUR confers metabolic sensing to the  $K_{ATP}$  channel complex by serving as a binding site for adenine nucleotides (12, 13), it is unknown whether intrinsic properties of this protein promote nucleotide exchange and thereby contribute to channel gating.

SUR, including the cardiac SUR2A isoform, possess two nucleotide binding domains, NBD1 and NBD2, located between the eleventh and twelfth transmembrane regions and at the carboxyl terminus of the protein (14, 15). A common feature of ABC proteins is that NBDs contain conserved Walker motifs that form nucleotide binding pockets (16–18). Mutations in these domains cause life-threatening diseases such as persistent hyperinsulinemic hypoglycemia of infancy or Tangier disease, a disorder of lipid metabolism (19–21). Mutations of key residues in SUR, which preclude nucleotide binding and/or hydrolysis in other ABC transporters (22, 23), alter the responsiveness of  $K_{ATP}$  channels to endogenous channel ligands, ATP and ADP (24, 25). These mutations also impede channel activation by  $K_{ATP}$  channel opening drugs (25–27). MgADP, interacting through NBDs, may stabilize an ‘activated’ state of SUR associated with a reduced sensitivity of the  $K_{ATP}$  channel complex to inhibition by ATP (18, 26, 28). Although it has been proposed that this SUR state could result from Mg ATP hydrolysis (18, 26, 28), such a catalytic function for NBDs in  $K_{ATP}$  channels has not been demonstrated.

We report that NBDs in SUR2A harbor an ATPase

CHANNEL FAMILIES ARE diversified through formation of heteromultimeric complexes, where distinct

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activity that determines the sensitivity of  $K_{ATP}$  channels to ATP and is modulated by potassium channel openers. This intrinsic property, reduced by mutations in Walker motifs, contributes to  $K_{ATP}$  channel activation by favoring catalytic conversion of nucleotides at the channel site. Assigning a catalytic activity to the sulfonylurea receptor subunit indicates that the cardiac  $K_{ATP}$  channel complex functions not only as a  $K^+$  conductance, but also as an enzyme regulating channel gating.

A preliminary account of this work has been published in abstract form (29).

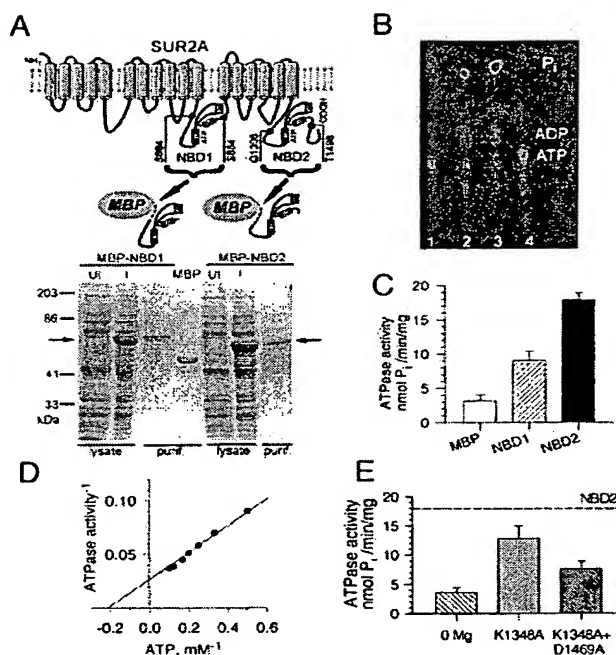
## MATERIALS AND METHODS

### ATPase activity in nucleotide binding domains

Nucleotide binding domains (Fig. 1A) NBD1 (Ser<sup>684</sup>-Ser<sup>884</sup>) and NBD2 (Gly<sup>1306</sup>-Thr<sup>1498</sup>) were amplified by polymerase chain reaction from rat SUR2A cDNA (15) and cloned in-frame with the maltose binding protein (MBP) coding sequence in pMal-c2 (New England Biolabs, Beverly, Mass.). The identity of fusion constructs was confirmed by DNA sequencing. NBDs were expressed in *Escherichia coli* TB1 induced with 0.1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) and fusion proteins were purified by affinity chromatography on an amylose resin in (in mM) 200 NaCl, 1 EDTA, 0.2 PMSF and 20 Tris (pH 7.4). Mutated NBDs (Lys<sup>1348</sup> to alanine and/or Asp<sup>1469</sup> to asparagine) were constructed in pMal-c2 by site-directed mutagenesis (QuickChange, Stratagene, San Diego, Calif.). ATPase activity of NBDs (10  $\mu$ g) was measured by monitoring production of [<sup>32</sup>P]P<sub>i</sub> from 1  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (30) in 34 mM KCl, 4 mM MgCl<sub>2</sub>, 50 mM HEPES (pH 7.4), and 4 mM ATP (4 h; 37°C). Using polyethylenimine cellulose thin-layer chromatography plates (Sigma, St. Louis, Mo.), nucleotides were resolved by ascending chromatography with 0.75 M KH<sub>2</sub>PO<sub>4</sub> and quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). ATPase activity was confirmed by measuring ADP formation using high-performance liquid chromatography (HPLC) and a spectrophotometric coupled enzyme assay (31).

### ATPase activity in $K_{ATP}$ channel immunoprecipitates

$K_{ATP}$  channels were immunoprecipitated from guinea pig heart membranes with a Kir6.2 antibody (32). Cardiac membranes (400  $\mu$ g) were solubilized in an immunoprecipitation buffer (IP in mM: 50 Tris-HCl, 150 NaCl, 5 EDTA, 50 NaF; pH 8.3) and incubated with a Kir6.2 antibody (raised in rabbit against amino acids N19-32C of rat Kir6.2). The resulting antibody/ $K_{ATP}$  channel complex was precipitated with protein A Sepharose. After washes in IP buffer with 1% Nonidet P-40, 1 mM PMSF, 10 mg/ml leupeptin, and phosphate-buffered saline (PBS), samples were centrifuged and resuspended in PBS buffer. The amount of SUR2A protein was calculated assuming a density of 5  $K_{ATP}$  channels/ $\mu$ m<sup>2</sup> of cardiac membrane (33) and an immunoprecipitation efficiency of 10% (34). To measure ATPase activity, channel immunoprecipitates or corresponding controls were incubated with 2 mM ATP and 2 mM MgCl<sub>2</sub> for 20 h at 37°C while shaken at 170 rpm. The reaction was stopped by HClO<sub>4</sub> (2 mM), and kept on ice for 5 min. Proteins were precipitated by centrifugation at 15,000  $g$  (4°C, 5 min). Supernates were neutralized with 2 M K<sub>2</sub>CO<sub>3</sub>; upon removal of potassium



**Figure 1.** ATPase activity in nucleotide binding domains of the  $K_{ATP}$  channel subunit SUR2A. A) NBD1 (Ser<sup>684</sup>-Ser<sup>884</sup>) and NBD2 (Gly<sup>1306</sup>-Thr<sup>1498</sup>) of SUR2A were subcloned into pMal-c2 plasmid in-frame with maltose binding protein (MBP). A and B: Walker A and B motifs, L: linker sequence. SDS/PAGE shows Coomassie blue-stained proteins from lysates of *E. coli* transformed with constructs containing MBP-NBD1 or MBP-NBD2. In contrast to uninduced cells (UI), induction by 1 mM IPTG (I) produced a prominent band corresponding to MBP-NBD1 (→) or MBP-NBD2 (←). Fusion proteins with NBD1 or NBD2 were purified (purif.) from lysates by affinity chromatography using an amylose resin. Purified MBP alone migrated at the expected size (43,000 kDa). B) ATPase activity in NBD1 and NBD2. The generation of [<sup>32</sup>P]P<sub>i</sub> from [ $\gamma$ -<sup>32</sup>P]ATP was analyzed by thin-layer chromatography, followed by autoradiography with an image analyzer. Lane 1: MBP; lane 2: MBP-NBD1; lane 3: MBP-NBD2; lane 4: blank. C) Average ATPase activity for MBP, MBP-NBD1, and MBP-NBD2 ( $n=6-8$  different preparations). D) Michaelis-Menten plot defining the ATPase activity of NBD2 ( $V_{max}$  35.4 nmol/min/mg and  $K_m$  of 4.4 mM). E) ATPase activity of NBD2 is diminished by removal of Mg<sup>2+</sup> from the incubation medium. Neutralization of the lysine (K1348A) in the Walker A motif of the binding fold alone or in combination with additional mutation of aspartate (D1469A) in the Walker B motif decreased the NBD2 ATPase activity.

perchlorate precipitate, adenine nucleotides were determined by HPLC (31).

### ATPase activity in cardiac membranes

Purified cardiac membranes were isolated as described (35). Hearts from guinea pigs (0.2-0.3 kg), anesthetized with pentobarbital (75 mg/kg), were homogenized in hypotonic buffer (in mM: 10 HEPES, 1 EGTA, 1 DTT, 1 aprotinin, 0.2 phenylmethylsulfonyl fluoride, and 1  $\mu$ g/ml leupeptin; pH 7.4) and spun at 5000  $g$  (4°C, 15 min). Supernatant was

centrifuged at 100,000 g (4°C, 1 h) and membrane pellets were suspended by sonication in (in mM) 20 HEPES (pH 7.4), 140 NaCl, 5 KCl, 2 MgCl<sub>2</sub>, 0.5 dithiothreitol, 1 aprotinin, 0.2 phenylmethylsulfonyl fluoride, and 2 µg/ml leupeptin. Sarcolemmal fraction was purified by sucrose density gradient centrifugation, and the degree of enrichment was determined based on Na, K-ATPase activity (36). Nascent ADP produced by ATPase activity was detected using a spectrophotometric coupled enzyme assay (31). Reaction medium contained (in mM) 50 Tris-HCl (pH 7.5), 50 KCl, 2 MgCl<sub>2</sub>, 2 dithiothreitol, 2 phosphoenolpyruvate, 0.15 NADH, 0.2 ouabain, 10 levamisole, 10 U/ml pyruvate kinase, 10 U/ml lactate dehydrogenase, and 10–20 µg membrane protein. Release of inorganic phosphate (P<sub>i</sub>), the other product of ATPase activity, was measured by spectrophotometry using an EnzChek Phosphate Assay kit (Molecular Probes, Eugene, Oreg.). Incubation medium contained (in mM) 50 Tris-HCl (pH 7.5), 0.2 MESG substrate, 2 ATP, 2 MgCl<sub>2</sub>, 2 dithiothreitol, 0.2 ouabain, 10 levamisole, and 5 U/ml purine nucleoside phosphorylase.

#### Recording of cardiac K<sub>ATP</sub> channels

Electrophysiological recordings were performed in ventricular myocytes dissociated from guinea pig hearts (37). Pipettes (~7–10 MΩ) were filled with (in mM) KCl 140, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, HEPES-KOH 5 (pH 7.3). For the inside-out configuration, cells were superfused with 'internal solution' (in mM): KCl 140, MgCl<sub>2</sub> 1, EGTA 5, HEPES-KOH 5 (pH 7.3). For the open cell-attached patch, 'internal solution' was supplemented with glucose (1 g/l), malic acid (5 mM), and pyruvic acid (5 mM). After seal formation, the open cell-attached configuration was obtained by applying digitonin (8 µg/ml) through a second pipette (filled with 5 µg/ml propidium iodide and 0.5 µg/ml rhodamine). Solution flow was visualized by rhodamine under ultraviolet light; staining the cell nucleus with propidium iodide served as a criterion for sarcolemmal permeabilization. For whole-cell recording, pipettes (~5 MΩ) were filled with 'internal solution' plus 4 mM ATP, and cardiomyocytes superfused with Tyrode (in mM): NaCl 136.5, KCl 5.4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 0.53, glucose 5.5, HEPES-NaOH 5.5 (pH 7.4). Whole-cell currents were obtained in response to 1 s rectangular pulses from a holding potential of -50 mV to 0 mV. Channel activity was expressed as NP<sub>o</sub>, where N represents the number of channels and P<sub>o</sub> the open channel probability. Concentration-dependent relationships were expressed in relative terms as NP<sub>o</sub> values measured in the presence *vs.* absence of a channel inhibitor and fitted with the Hill equation (37). Single-channel analysis was performed as described (37, 38).

#### Recording of recombinant K<sub>ATP</sub> channels

Kir6.2 (5) and SUR2A (15), subcloned into a pGEMHE vector (25), were amplified and transcribed using the T7 mMessage mMachine kit (Ambion, Austin, Tex.). Mutagenesis of Lys<sup>1348</sup> to methionine or Asp<sup>1469</sup> to asparagine in SUR2A was done in the pGEMHE-SUR2A plasmid (QuickChange, Stratagene). cRNAs coding Kir6.2 (2 ng) and SUR2A (6 ng) were coinjected into defolliculated *Xenopus laevis* oocytes (25, 39). Recombinant K<sub>ATP</sub> channel currents were subsequently recorded in inside-out membrane patches (25, 39) using pipettes (2–10 MΩ) containing (in mM) 154 K<sup>+</sup>, 146 Cl<sup>-</sup>, 5 Mg<sup>2+</sup>, and 10 PIPES-KOH (pH 7.1). The cytoplasmic face of the patch was bathed in (in mM) 174 K<sup>+</sup>, 40 Cl<sup>-</sup>, 1 Mg<sup>2+</sup>, 1 EGTA, 10 PIPES-KOH (pH 7.1), and methanesulfonate<sup>-</sup>. Membrane potential was maintained at -50 mV.

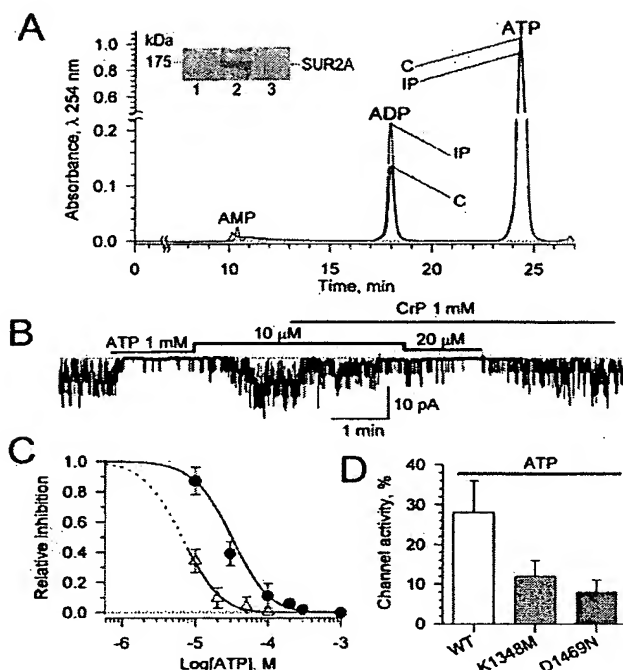
#### Statistical analysis

Results are expressed as mean ± SE; *n* refers to the number of samples from different preparations used in each analysis. Significant differences for unpaired samples were assessed by Student's *t* test. Difference at *P* < 0.05 was considered significant.

## RESULTS

#### ATPase activity in nucleotide binding domains of the sulfonylurea receptor

Nucleotide binding domains of the sulfonylurea receptor SUR bind ATP (18). NBD1 and NBD2 of the SUR2A isoform were engineered in-frame with the MBP to facilitate purification (Fig. 1A). *E. coli*, transformed with respective constructs and induced with IPTG, expressed a high yield of NBD1 and NBD2 fusion proteins detected at corresponding molecular weights on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE; Fig. 1A). NBDs purified by affinity chromatography were exposed to γ-labeled [<sup>32</sup>P]ATP and ATPase activity was measured by monitoring generation of [<sup>32</sup>P]P<sub>i</sub> (Fig. 1B). The estimated ATPase activity was 9.1 ± 0.6 and 17.9 ± 1.0 nmol P<sub>i</sub>/min/mg for NBD1 (*n*=5) and NBD2 (*n*=7) fusion constructs, respectively (Fig. 1C). These values were significantly (*P* < 0.01) different between each other and higher than 3.2 ± 0.9 nmol P<sub>i</sub>/min/mg (*n*=7) measured in samples containing MBP alone (Fig. 1C). The ATPase activity of NBD1 and NBD2 was further confirmed by following ADP formation using HPLC and spectrophotometry (not illustrated). Either approach consistently demonstrated a significantly higher ATPase activity in NBD2 compared to NBD1 fusion proteins. The ATPase activity in NBD-containing samples was insensitive to inhibitors of conventional ATPases and phosphatases, ouabain (1 mM), oligomycin (10 µg/ml), thapsigargin (0.1 mg/ml), and/or levamisole (10 mM). The ATPase activity of NBDs was dependent on ATP concentration (Fig. 1D). For the most active NBD2, the Michaelis-Menten plot exhibited a V<sub>max</sub> of 35.4 nmol/min/mg and an apparent K<sub>m</sub> of 4.4 mM (Fig. 1D). Removal of Mg<sup>2+</sup>, a cofactor in the ATPase reaction, reduced the ATPase activity (Fig. 1E). Site-directed mutagenesis of the lysine residue (K1348A), in the signature Walker A motif of NBD2, reduced ATPase activity (Fig. 1E). This catalytic activity was further diminished in the double NBD2 mutant in which the Walker A lysine<sup>1348</sup> moiety was mutated in combination with the Walker B aspartate<sup>1469</sup> (K1348A+D1469N). Thus, nucleotide binding domains in the sulfonylurea receptor, primarily NBD2, possess intrinsic ATPase activity.



**Figure 2.** ATPase activity of the  $K_{ATP}$  channel complex. *A*) HPLC chromatogram showing increased ADP generation from ATP in cardiac sarcolemmal Kir6.2-immunoprecipitates (IP) compared to preimmune control serum (C). Inset: Immunoblot of cardiac sarcolemma probed with a monoclonal SUR2A antibody in the absence (lane 1) or after immunoprecipitation by a Kir6.2 antibody (lane 2). In the control lane 3, the secondary rabbit-IgG-HRP antibody cannot recognize a protein in Kir6.2 immunoprecipitates in the absence of the primary SUR2A antibody. *B*) Activation of the creatine kinase-catalyzed, ADP-removing system by creatine phosphate (CrP) increases the sensitivity of native cardiac  $K_{ATP}$  channels. Current record was obtained in open cell-attached patches at a holding potential of  $-60$  mV. *C*) Concentration-response curve for ATP-induced inhibition of cardiac  $K_{ATP}$  channels in inside-out patches in the absence of the CrP/creatine kinase system (filled circles) and in open cell-attached patches with  $1$  mM CrP (open circles). *D*) Sensitivity of recombinant wild-type and mutant cardiac  $K_{ATP}$  channels toward ATP ( $100$   $\mu$ M). Wild-type Kir6.2/SUR2A ( $n=8$ ) was inhibited by 70% relative to channel activity in the absence of ATP, whereas Kir6.2 coexpressed with mutant K1348M ( $n=6$ ) or D1469N ( $n=6$ ) SUR2A was inhibited by roughly 90% of control activity. Currents in each condition were obtained in inside-out patches after expression in oocytes.

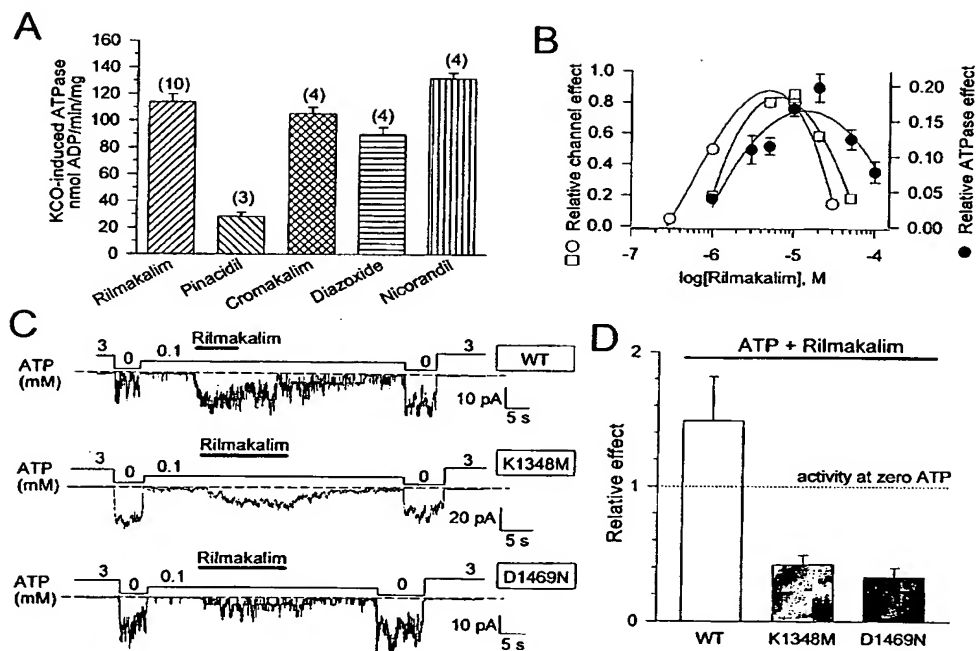
#### ATPase activity of the $K_{ATP}$ channel complex regulates channel sensitivity to ATP

SUR2A associates with Kir6.2 to form cardiac  $K_{ATP}$  channels (15, 32). Accordingly, a Kir6.2 antibody (11) coimmunoprecipitates both Kir6.2 and SUR2A subunits from cardiac sarcolemma (Fig. 2A, inset). In immunoprecipitates of  $K_{ATP}$  channel proteins, ATPase activity was assayed by HPLC (Fig. 2A). Consistent with enzymatic activity of  $K_{ATP}$  channels, immunoprecipitates of the channel complex con-

verted ATP into ADP, with an ATPase activity estimated at  $31 \pm 8$  nmol ADP/min/mg ( $n=7$ ). Such ATPase activity could catalyze local hydrolysis of ATP, and thereby promote  $K_{ATP}$  channel opening. Indeed,  $10$   $\mu$ M ATP failed to inhibit native cardiac  $K_{ATP}$  channels (40) under conditions of unimpeded ATPase activity (Fig. 2B). However, when the product of the ATPase reaction, ADP, was continuously removed through creatine phosphate (CrP)-activated creatine kinase (41),  $10$   $\mu$ M ATP suppressed  $K_{ATP}$  channel opening (Fig. 2B). In fact, in the presence of CrP, the  $IC_{50}$  for channel inhibition was reduced by over threefold, from  $25.1 \pm 1.4$   $\mu$ M ( $n=9$ ) to  $7.4 \pm 0.4$   $\mu$ M ( $n=4$ ; Fig. 2C). Moreover, mutations of the Walker A lysine<sup>1348</sup> and Walker B aspartate<sup>1469</sup>, which reduce ATPase activity of NBD2 (Fig. 1E), produced recombinant  $K_{ATP}$  channels with a higher sensitivity to ATP ( $100$   $\mu$ M) compared to wild-type Kir6.2/SUR2A (Fig. 2D). Thus, the ATPase activity of  $K_{ATP}$  channels regulates the channel responsiveness to ATP.

#### Potassium channel opener-induced ATPase activity

Potassium channel openers bind to SUR and promote  $K_{ATP}$  channel opening by reducing channel sensitivity to ATP (39, 42–45). In cardiac sarcolemma, the structurally distinct potassium channel openers rilmakalim ( $10$   $\mu$ M), pinacidil ( $50$   $\mu$ M), cromakalim ( $100$   $\mu$ M), diazoxide ( $100$   $\mu$ M), and nicorandil ( $100$   $\mu$ M) promoted hydrolysis of ATP into ADP, indicating activation of ATPase activity (Fig. 3A). Depending on the potassium channel opener tested, opener-induced ATPase activity ranged from  $28$  to  $132$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup> above the basal membrane ADP-generating capacity (Fig. 3A). Opener-induced ADP generation was associated with increased liberation of inorganic phosphate, the other product in the ATPase reaction. In the presence of a representative potassium channel opener, rilmakalim ( $10$   $\mu$ M), the ATPase activity calculated by increased inorganic phosphate ( $P_i$ ) generation was  $139 \pm 8$  nmol  $P_i$   $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup> ( $n=6$ ), a value similar to that obtained from ADP-generation measurements ( $114 \pm 6$  nmol ADP  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>;  $n=10$ ). Opener-induced increase in ATPase activity required  $Mg^{2+}$ . However, it was not inhibited by ouabain ( $200$   $\mu$ M), oligomycin ( $1$   $\mu$ g/ml), and/or levamisole ( $10$  mM). Corresponding rilmakalim-induced ATPase activities were  $139 \pm 10$ ,  $119 \pm 8$ , and  $118 \pm 9$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup> with ouabain ( $n=4$ ), oligomycin ( $n=4$ ) and levamisole ( $n=4$ ) alone, and  $111 \pm 6$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup> ( $n=4$ ) in the presence of a mixture containing all three conventional ATPase and phosphatase inhibitors. This suggests that the effect of openers on ADP and  $P_i$  generation is not due to activation of sarcolemmal Na,K-ATPase, mitochondrial  $F_1F_0$ -ATPase, or alkaline



**Figure 3.** Potassium channel openers modulate ATPase activity. *A*) Potassium channel opener (KCO)-activated ATPase activity in cardiac sarcolemma. Rilmakalim (10  $\mu$ M), pinacidil (50  $\mu$ M), cromakalim (100  $\mu$ M), diazoxide (100  $\mu$ M) and nicorandil (100  $\mu$ M) increased ATPase activity measured as ADP generation using a spectrophotometric method. Basal sarcolemmal ATPase activity was  $646 \pm 9$  nmol ADP  $\cdot$  min $^{-1} \cdot$  mg protein $^{-1}$  ( $n=10$ ). Number of experiments for each opener is shown in parentheses. *B*) Bell-shaped concentration-dependent relationship of rilmakalim-mediated activation of ATP hydrolysis. Opener-induced channel activity was measured in whole-cell (open squares) and inside-out (open circles) patches, and expressed relative to the left scale. Opener-induced ATPase activity (filled circles) was estimated by ADP generation and expressed relative to the right scale. *C*) Reduced effect of rilmakalim on recombinant cardiac K<sub>ATP</sub> channels with mutations suppressing ATPase activity. Current traces obtained from oocytes coexpressing Kir6.2 with wild-type SUR2A (WT) or SUR2A mutants K1348M or D1469N. *D*) Average values of patch current measured in the presence of ATP (100  $\mu$ M) before and after application of rilmakalim (10  $\mu$ M) for wild-type ( $n=8$ ), K1348M ( $n=6$ ), and D1469N ( $n=6$ ) SUR2A. Currents in each condition were obtained in inside-out patches, and normalized to the current in the absence of ATP obtained by linear extrapolation of values measured before and after opener application.

phosphatase. In contrast, 10  $\mu$ M rilmakalim failed to promote ATPase activity in the presence of 10 mM azide, an inhibitor of ATPase activity in purified ABC proteins (46). The effect of openers on cardiac membrane ATPase activity was concentration dependent; with rilmakalim, maximal activation was observed between 10 and 20  $\mu$ M (Fig. 3*B*). Beyond this concentration the opener was less effective (Fig. 3*B*), and at 200 to 500  $\mu$ M could inhibit membrane ATPase activity by 15 to 35%. Within the concentration range from 0.1 to 100  $\mu$ M, the bell-shaped dependence of rilmakalim-induced ATPase activity closely correlated with the concentration dependence of K<sub>ATP</sub> channel activation (Fig. 3*B*). Mutations in SUR2A that suppressed ATPase activity (Fig. 1*E*) also reduced the ability of rilmakalim (10  $\mu$ M) to activate K<sub>ATP</sub> channels inhibited by ATP (Fig. 3*C, D*). Rilmakalim vigorously activated wild-type Kir6.2/SUR2A, and was four- to fivefold less effective after mutations in Walker A lysine<sup>1348</sup> and Walker B aspartate<sup>1469</sup>, respectively (Fig. 3*C, D*). Thus, potassium channel openers modulate ATPase activity and muta-

tions that reduce such catalytic activity reduce opener-mediated channel activation.

#### Openers mimic ADP-induced K<sub>ATP</sub> channel behavior

At the single-channel level, the product of the ATPase reaction, ADP, induces a readily recognizable pattern of K<sub>ATP</sub> channel transitions between open and closed conformations (37). ADP (0.1 mM) had no effect on intraburst transitions ( $C_1 \leftrightarrow O$ ; rates  $k_{01}$  and  $k_{10}$ ), but slowed burst closure (rate  $k_{02}$ ) and diminished lifetime in long-lasting ( $C_2 \leftrightarrow C_3$ ) closed states (accelerated rates  $k_{20}$  and  $k_{32}$ , and reduced rate  $k_{23}$ ; Fig. 4*A, B*). This pattern was associated with burst prolongation (from 1.3 s in the absence to 3.9 s in the presence of ADP) and shortened interburst events leading to increased open channel probability (from 0.22 to 0.62; Fig. 4*A*). Similarly to MgADP, rilmakalim (10  $\mu$ M) and pinacidil (50  $\mu$ M) also did not affect intraburst transitions (rates  $k_{01}$  and  $k_{10}$ ;

**Figure 4.** ADP and potassium channel openers produced similar changes in  $K_{ATP}$  channel kinetics. *A*) Representative  $K_{ATP}$  channel records measured at +60 mV holding pipette potential. Four states kinetic schemes, with calculated rates of transition, are presented on the right of each corresponding channel record. *B*) ADP and potassium channel openers (rilmakalim and pinacidil) produced similar changes in transition rates defining  $K_{ATP}$  channel kinetic behavior. Changes in rates produced by ADP and openers are presented as a relative slowing with regard to values obtained in the presence of ATP alone ( $n=3$  for each experimental condition).

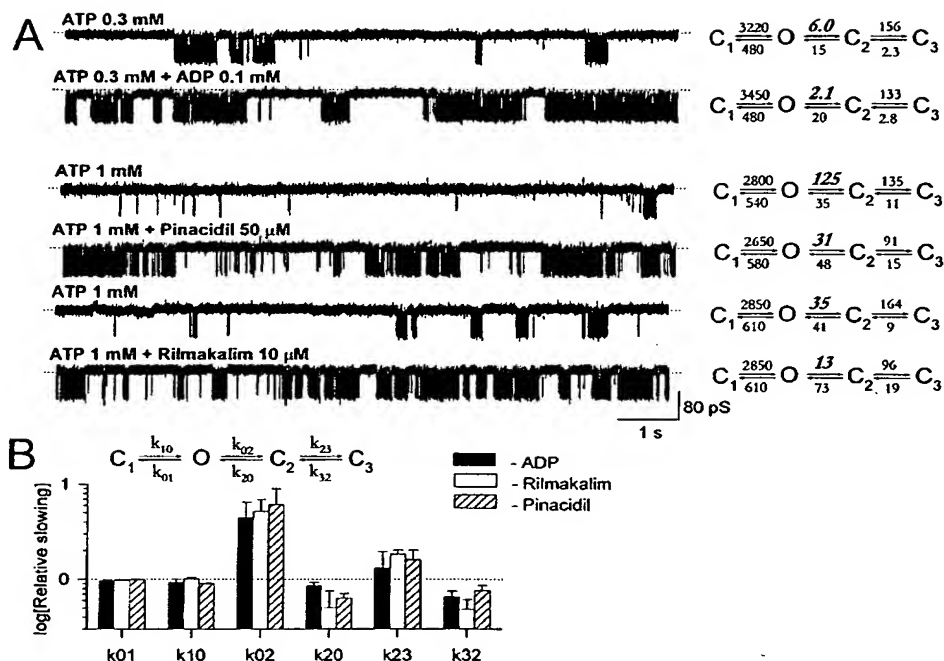


Fig. 4A, B). Like ADP, both openers slowed  $k_{02}$  and  $k_{23}$  while accelerating  $k_{20}$  and  $k_{32}$  (Fig. 4A, B). This prolonged burst duration (from 0.17 to 0.46 s for rilmakalim and from 0.05 to 0.17 s for pinacidil) shortened interburst events and increased open channel probability (from 0.28 to 0.84 and from 0.11 to 0.54, respectively). Thus, openers and MgADP induce the same profile of  $K_{ATP}$  channel conformational transitions associated with channel activation.

#### Creatine kinase regulates opener-induced $K_{ATP}$ channel opening

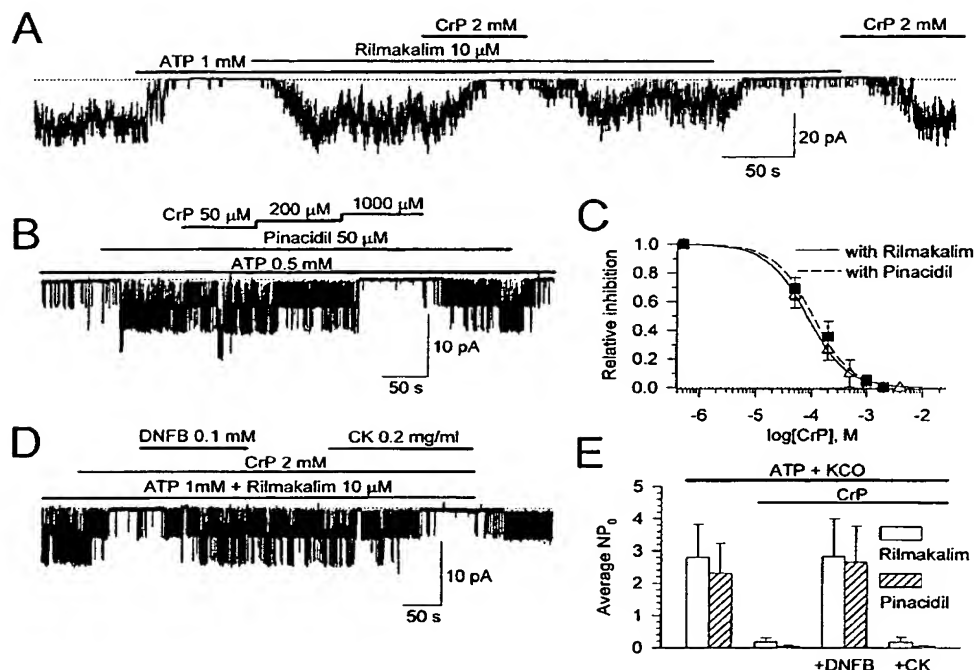
Creatine kinase, which catalyzes ADP phosphorylation in the  $MgADP + CrP \leftrightarrow creatine + MgATP$  reaction, provides the major phosphotransfer pathway in the heart (31). After sarcolemmal permeabilization, which results in loss of cellular CrP and creatine kinase-dependent ADP utilization, millimolar concentrations of ATP were required to inhibit  $K_{ATP}$  channels (Fig. 5A). Under such conditions, rilmakalim (10 μM; Fig. 5A) and pinacidil (50 μM) readily reversed ATP-inhibited channel activity. On average,  $K_{ATP}$  channel activity in the presence of openers ( $NP_0 = 4.9 \pm 0.6$ ;  $n=35$  and  $4.5 \pm 0.9$ ;  $n=15$ , respectively) was similar to that obtained in the absence of ATP ( $4.9 \pm 0.4$ ;  $n=53$ ). In fact, more than 10 mM ATP was required to abolish rilmakalim (10 μM) or pinacidil (50 μM)-induced channel opening. However, the effect of openers was abolished by supplying CrP (Fig. 5A, B). CrP antagonized the effect of distinct openers with the same potency (Fig. 5C), suggesting a common mechanism, such as ADP

removal, as responsible for inhibition of rilmakalim- and pinacidil-induced channel activity. This effect of CrP was abolished by 0.1 mM 2-4-dinitrofluorobenzene (DNFB), an irreversible creatine kinase inhibitor (Fig. 5D, E). Application of exogenous creatine kinase (0.2 mg/ml) restored the ability of CrP to suppress opener-induced  $K_{ATP}$  channel activity (Fig. 5D, E). Thus, the efficacy of potassium channel openers to activate  $K_{ATP}$  channels is determined by the availability of a reaction system capable of scavenging ADP, the product of the ATPase reaction.

#### DISCUSSION

This study provides direct evidence that the cardiac  $K_{ATP}$  channel harbors an ATPase activity sensitive to potassium channel openers. This catalytic activity was identified in NBD2 and, to a lesser extent, in NBD1 domains of the SUR2A subunit. Mutations in NBD2 that reduced the ATPase activity increased the sensitivity of  $K_{ATP}$  channels to ATP and attenuated the ability of openers to activate  $K_{ATP}$  channels. This intrinsic enzymatic activity may thus contribute to the nucleotide- and opener-dependent gating of  $K_{ATP}$  channels.

In some ABC proteins, NBDs are known to contain ATPase activity critical for protein function (47–50). The ATPase intrinsic to ABC transporters has been proposed to serve as a switch between ATP- and ADP-liganded conformations and the energy of ATP hydrolysis implicated in supporting transport function and ion conductance (17, 51). The SUR subunit



**Figure 5.** Creatine phosphate/creatine kinase inhibits opener-induced  $K_{ATP}$  channel activity. *A*) Opener-activated, ATP-inhibited  $K_{ATP}$  channels in a cardiomyocyte. Calculated (not shown) concentration dependence for ATP-induced channel inhibition ( $K_i = 0.27 \pm 0.02$  mM;  $h = 2.1 \pm 0.27$ ) was significantly shifted to the right by 10  $\mu$ M rilmakalim ( $K_d = 5.7 \pm 0.5$  mM;  $h = 2.4 \pm 0.5$ ) or 50  $\mu$ M pinacidil ( $K_i = 2.4 \pm 0.1$  mM;  $h = 2.3 \pm 0.2$ ). Creatine phosphate (CrP) inhibited the effect of opener. Alone, CrP did not significantly inhibit  $K_{ATP}$  channel activity. *B*) CrP inhibited opener-induced  $K_{ATP}$  channel activity in a concentration-dependent manner. *C*) The concentration-dependent relationship defining the CrP effect was indistinguishable in the presence of rilmakalim (open triangles, solid curve) or pinacidil (filled squares, dashed curve). Curves were constructed using a fit of experimental data ( $n = 3$  for each opener) by the Hill equation and characterized by a  $K_i = 83 \pm 2$   $\mu$ M ( $h = 1.2 \pm 0.1$ ) with rilmakalim (10  $\mu$ M) and  $107 \pm 9$   $\mu$ M ( $h = 1.1 \pm 0.1$ ) with pinacidil (50  $\mu$ M). *D*) DNFB, a potent inhibitor of creatine kinase, antagonized CrP inhibition of opener-induced  $K_{ATP}$  channel activity. Application of purified creatine kinase (CK) reversed the effect of DNFB. *E*) Average  $NP_0$  values obtained in the presence of ATP (1 mM) plus a potassium channel opener (10  $\mu$ M rilmakalim or 50  $\mu$ M pinacidil) as indicated under control conditions in the presence of creatine phosphate (CrP, 2 mM), CrP plus DNFB (0.1 mM), and CrP plus purified creatine kinase (0.2 mg/ml). Data were averaged from five experiments under each condition.  $K_{ATP}$  channel activity was measured in cardiomyocytes, using the open cell-attached patch configuration, at a holding pipette potential of  $-60$  mV.

of the  $K_{ATP}$  channel has been recognized for its role in channel trafficking and biogenesis (52, 53), as a receptor for pharmacological modulators (39, 43, 44), and as a site for nucleotide binding (12, 13, 18, 28, 54). The present demonstration of ATPase activity in NBDs identifies a new property for SUR, supporting previous suggestions that such catalytic activity could serve as an underlying mechanism for the nucleotide regulation of  $K_{ATP}$  channels (18, 26, 28, 55).

The  $V_{max}$  and  $K_m$  for the ATPase in NBDs of SUR2A were within the range of values reported for ATPase activity of other ABC proteins (47, 56). As in other ABC transporters (57), the  $K_{ATP}$  channel ATPase activity was insensitive to inhibitors of F- or P-ATPase types, indicating that the SUR ATPase is distinct from conventional ATPases. Nevertheless, it required  $Mg^{2+}$ , confirming the catalytic nature of ATP hydrolysis. Although mutations in conserved Walker motifs did not completely abolish ATPase

activity, the rate of ATP hydrolysis was significantly decreased, particularly for the double mutant K1348A+D1469N where amino acids in both Walker A and B were neutralized. Equivalent site-directed mutagenesis in Walker motifs of other ABC transporters also reduce their respective ATPase activities (47, 58, 59).

Suppression of ATPase activity by mutations in Walker motifs of SUR2A increased the sensitivity of cardiac  $K_{ATP}$  channels to ATP, as in the mutated SUR1 isoform (24). Furthermore, neutralizing the product of ATP hydrolysis, by the CrP/creatine kinase phosphotransfer system, revealed a threefold higher  $K_{ATP}$  channel sensitivity to ATP (60). Thus, the intrinsic ATPase activity of  $K_{ATP}$  channels sets the apparent ATP-sensitivity of the channel to a level lower than that expected in the absence of ATP hydrolysis.

Potassium channel openers, which specifically bind to SUR (39, 43, 44), promoted ATPase activity.



The concentration dependence of opener-induced ATPase activity closely correlated with opener-induced  $K_{ATP}$  channel activation. At higher concentrations, potassium channel openers inhibited ATPase activity while still producing  $K_{ATP}$  channel activation (61). This apparent contradiction can be related to an opener-induced stabilization of MgADP in the active ATPase site after ATP hydrolysis (29). ADP trapping at the ATPase active site observed with conventional ATPase inhibitors (48) would keep the channel predominantly in the ADP-bound state, thereby promoting channel opening. As openers mimicked MgADP-induced  $K_{ATP}$  channel kinetic behavior, this would support the proposed concept that activation of  $K_{ATP}$  channels may be associated with ADP production and/or stabilization of the ADP-bound state at the SUR subunit (26, 28, 55).

Mutations of conserved lysine to methionine (K1348M) and aspartate to asparagine (D1469N) residues in Walker A and B motifs of NBD2, which abolish channel activation by ADP (25), reduced rilmakalim-induced channel opening. This may be associated with partial inhibition of ATPase activity observed with such mutations or with the possibility that openers could act through an alternative pathway, including an ADP-independent mechanism. This is supported by the observation that analogous mutations in the SUR1 isoform abolished the effect of ADP, but only reduced the action of openers or metabolic stress on channel activity (27).

Potassium channel opener-induced  $K_{ATP}$  channel opening was inhibited by the CrP/creatine kinase system, which removes ADP from the channel site. Under this condition, due to relief of end-product inhibition, the ATPase reaction should proceed at an even higher rate (41, 62). Thus, the product of ATPase catalysis, ADP, rather than ATP hydrolysis *per se*, appears to be essential for channel activation. By scavenging the ATPase product, creatine kinase would provide an efficient means of regulating  $K_{ATP}$  channel behavior (41, 60, 63). In the heart, creatine kinase is the major phosphotransfer system whose flux is dramatically reduced under metabolic stress (31, 62). Here, inhibition of creatine kinase promoted  $K_{ATP}$  channel activation by openers. The reported higher responsiveness of ischemic hearts to openers (64) could be the consequence of reduced creatine kinase flux early in ischemia, which would facilitate activation of  $K_{ATP}$  channels and associated cardioprotective processes (65). Thus, the balance between ATP hydrolysis, through the opener-sensitive channel ATPase, and ADP removal, through the creatine kinase system, provides an integral mechanism of  $K_{ATP}$  channel regulation under different cellular metabolic states.

It should be noted that removal of ADP by creatine kinase is associated with ATP generation, which, if

sufficient, could inhibit opener-induced channel activation. Inhibition of opener-induced  $K_{ATP}$  channel activity would require ~10 mM of ATP. Under our experimental conditions, such elevation of ATP is unlikely since the source for ATP regeneration is ADP resulting from intracellular ATP hydrolysis, and therefore the overall concentration of synthesized ATP, cannot far exceed 1 mM of ATP applied in the bath solution. Although activation of an ATP-regenerating system, which removes ADP, may reduce opener binding to the cardiac SUR2A isoform (66), this has not been observed at concentrations of nucleotides used in our experiments. Thus, loss of opener-induced channel activation in the presence of an ADP-scavenging system is apparently not due to an increase in the local ATP concentration or reduction of opener binding.

In summary, this study demonstrates that the cardiac  $K_{ATP}$  channel complex possesses an ATPase activity found in NBDs of the SUR2A subunit. Such intrinsic enzymatic activity defines  $K_{ATP}$  channels not only as passive targets responding to alterations in the cellular metabolic status, but also as active contributors to their nucleotide-dependent gating. Therefore, modulation of the channel ATPase activity and/or of metabolic systems that scavenge the product of the ATPase reaction provides a novel means of regulating vital cellular functions associated with  $K_{ATP}$  channel opening. F

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